Title: Expression pattern of miR-451 and its target *MIF (macrophage migration inhibitory factor)* in colorectal cancer

Running head: miR-451 and MIF in colorectal cancer

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Abstract

AIMs: To investigate the expression pattern of miR-451 in patients with colorectal carcinoma and correlate with the expression of its target gene *MIF* (*macrophage migration inhibitory factor*).

METHODS: Matched cancer and non-cancer fresh frozen tissue were prospectively collected from 70 patients (35 men and 35 women) underwent resection of colorectal adenocarcinoma. These tissues collected were extracted for microRNA and cDNA conversion. Then, miR-451 expressions in these tissues were measured by quantitative real-time polymerase chain reaction (qRT-PCR). The expression was correlated with clinical and pathological parameters of these patients. In addition, paraffin blocks of 10 colorectal carcinomas with lowest expression of miR-451 were used for the study of MIF protein expression by immunohistochemistry.

RESULTS: miR-451 was downregulated in majority of the colorectal cancer tissues when compared to their matched normal tissues (84.3%, n=59/70). Downregulation of miR-451 correlates significantly with presence of co-existing adenoma (91.4%, p=0.025). In addition, persistence of cancer or cancer recurrence after surgery showed significant correlation with downregulation of miR-451 (80% versus 0%; p=0.028). There is no significant correlation between miR-451 expression and age, gender of the patients as well as size, grades, pathological stages, presence of lymphovascular permeation, peri-neural invasion and MSI status of the colorectal carcinoma (p > 0.05). Majority of the cases (80%) with low expression of miR-451 showed high levels of MIF protein expression confirming the inverse relationship between miR-451 and MIF expressions.

CONCLUSION: The results showed that miR-451 could play a role in development and progression of colorectal cancer and likely by targeting *MIF*.

INTRODUCTION

MicroRNAs (miRs) are important in the pathogenesis and have potential applications in colorectal carcinoma.[1-5] Amongst these, miR-126, miR-1288 and miR-498 have shown to be shown in our previous studies to be important in the pathogenesis of colorectal carcinoma by analysing large cohort of clinical samples of the cancer.[2-4] In addition, studies have shown that other miRNAs, in particular miR-21, miR-200, miR-215 are commonly involved in regulation of cancer stem cells in colorectal carcinoma.

Amongst the microRNAs, miR-451 has important roles in pathogenesis of colorectal cancer and with downstream target identified. miR-451 is located on chromosome 17 at 17q11.2 of human genome.[6] High expression level of miR-451 was reported in human foetuses when compared to adults indicating that miR-451 could play important role in cell growth and differentiation. [7,8] miR-451 acts as a tumour suppresser gene in modulating different molecular pathways in many cancers. [9-14] To exert its effects, miR-451 target several genes and most significant target is *MIF* (*macrophage migration inhibitory factor*).[15-16] MIF protein is a pro-inflammatory cytokine that has association with pathogenesis of chronic inflammatory diseases,[17] and many types of cancers.[18-19] Recent studies have proven that MIF has the potential to act as target against cancer as its expression in human cancers correlates positively with the tumour aggressiveness and metastatic potential.[20-22]

Till now, tumour suppressive properties of miR-451 in colorectal carcinoma have been studied mostly on *in vitro* analysis. Moreover, previous studies did not correlate the co-expression pattern of miR-451 and its target *MIF* in cancer tissues. Thus, this study aims to analyse the role of miR-451 in colorectal carcinoma by identification its expression pattern in conjunction with MIF protein expression status and correlate this expression with different clinicopathological parameters of the patients with colorectal carcinoma.

MATERIALS AND METHODS

Recruitment of the tissues and sample selection

A total of 70 fresh frozen colorectal cancer tissue specimens with matched non-cancer tissues were prospectively recruited and stored in -80°C. The samples were collected from patients with colorectal carcinoma who have undergone resection. A signed consent from each of the participating patients was also obtained. Formalin fixed paraffin tissue blocks from the same tissues were also obtained for MIF protein expression analysis. The fresh frozen colorectal tissues were sectioned using a cryostat (Leica Biosystems, Wetzlar, Hesse, Germany) and histological analysis was made after staining with hematoxylin and eosin staining. This study was approved by the Human Research Ethics Committee of Griffith University (GU Ref No: MSC/17/10/HREC).

Clinical and pathological data of the patients

The surgical specimens of colorectal cancer were processed for pathological examination. All the histological sections of the colorectal cancer were examined by the author (AKL).

There are 70 patients (35 men and 35 women) with colorectal adenocarcinoma collected. The mean age of the patients was 70 years (range from 24-91years). The size of the cancer was classified into two groups - less than 40 mm (n= 39) and more or equal to 40 mm (n=31). Fifty-four carcinomas were located in colon and 16 were in the rectum. The cancer was graded according to the World Health Organization (WHO).[23] Staging of the cancer was done according to TNM (Tumour, Lymph node, Metastasis) classification.[24]

The other pathological parameters including the association of the colorectal adenoma, presence of lymphovascular permeation, peri-neural invasion and hereditary nonpolyposis colorectal cancer (HNPCC) were also noted. Microsatellite Instability (MSI) was tested in these cases by immunohistochemistry on 4 proteins (MLH1, PMS2, MSH2 and MSH6) as described previously.[2]

All the patients were follow-up clinically according to standard protocol. The management of the patients were determined in the multi-disciplinary team

meeting. Radiological examination, endoscopic examination and haematological examination were offered to the patients by standard protocol and according to the clinical status of the patient. The patients were reviewed regularly by the surgical and/or oncology teams. Persistence (presence of residual tumour or metastases) or recurrence of the cancer after surgery was recorded. If patients have no persistent disease or cancer recurrence after 5 years, they were then referred to be followed up by their general practitioners.

MicroRNA extraction and cDNA conversion

Total RNA including miRNA was extracted from the fresh frozen human tissues after cryotomy. In brief, tissues were sectioned in thin slices (5µm) and followed by extraction using Qiagen miRNeasy Mini Kit which is specific for purification the total RNA including miRNA from fresh frozen tissues (Qiagen, Hilden, North Rhine-Westphalia, Germany). RNA quality was assessed by using a Nano Drop ND.1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, MA, U.S.A.). Purity of RNA was obtained by checking the optical density (OD) 260/280 ratio and concentration of RNA was noted in ng/µl.

Reverse transcription reactions were performed using 1 µg total RNA in a final reaction volume of 20 µl. RNA was converted to cDNA using miScript Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Each cDNA sample was diluted to 1.5 ng/µl in order to provide uniformly concentrated sample for miRNA quantitative real time polymerase chain reaction. The sample was stored at -20°C till the PCR analysis.

Quantitative Real-time PCR

An IQ5 Multicolour Real-Time PCR Detection system (Bio-Rad, Hercules, CA, USA) was used to analyse the miR-451 expression changes. PCR was performed in total volume of 20 μ l reaction mixture containing 10 μ l 2x QuantiTect SYBR Green PCR Master Mix, 2 μ l 10x miScript Universal Primer, 2 μ l 10x miScript Primer Assay (Hs_miR-451_1)(Qiagen) and 4 μ l of cDNA template at 1.5 ng/ μ l concentration. RNU6B RNA (Hs_RNU6B_2 miScript Primer Assay, Qiagen) was used for normalizing the amount of target miRNA. The primer sequences used for miR-451a and RNU6B were 5'AAACCGUUACCAUUACUGAGUU and 5'TGACACGCAAATTCGTGAAG respectively (Qiagen). All the samples (cancer and non-cancer) were run in triplicates. The fold change in the target gene for the results of quantitative amplification was calculated for each sample using $2^{-\Delta\Delta CT}$ (fold change) method as previously reported. [2-4] A fold change more than 2 was considered as high miR-451 expression and a fold change of less than 1 was regarded as low expression for miR-451.

Immunohistochemistry of MIF in colorectal cancer tissues:

Tissues from 10 colorectal adenocarcinoma patients with lowest expression of miR-451 noted in the previous experiment were used for MIF protein expression analysis. A high PH ENVision™ FLEX Mini Kit (Dako, Glostrup, Hovedstaden, Denmark) was used for the immunohistochemical analysis of MIF protein. In brief, deparaffinised and rehydrated sections (4 μ m) were first processed for antigen retrieval in citrate buffer (at pH 6.0) for 15 minutes in a microwave oven and then blocked with peroxidase blocking reagent for 8 minutes. Tissues were incubated with a rabbit antihuman MIF polyclonal antibody (FL-115, 1:150 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 60 minutes at room temperature. The slides were then incubated with a horseradish peroxidase conjugated rabbit secondary antibody (Dako) for 25 minutes at room temperature. Visualization of staining was done by using 3, 3'-diaminobenzidine (DAB) (Dako) to produce a brown colour, and then counterstained with haematoxylin. A colon adenocarcinoma tissue that showed strong nuclear staining was used as a positive control in each run

of the experiment. Negative control sample was prepared with the same procedure, except that tissue was not incubated with the primary antibody.

The staining was counted by a pathologist (AKL) under a standard light microscope. A grading scale ranging from 0 to 3 was used for this assessment, where 0 represented a negative staining, 1 represented weak staining (1-30%), 2 represented moderate staining (31 to 70%) and 3 represented strong staining (>70%). Assessment of the slides according to the extent of positive MIF staining was also taken in consideration.

Statistical analysis

Statistical analysis was done by using the Statistical Package for Social Sciences (IBM SPSS, version 23, New York, NY. USA). Chi-square test or likelihood ratio was used for categorical variables. Pearson correlation test was used for continues variables. Independent t-test was performed for the analysis of continues variables in categories value < 0.05 for these tests was considered as significant.

RESULTS

miR-451 expression pattern and correlation to clinicopathological parameters of the patients (Table 1)

MiR-451 expression was down-regulated in colorectal carcinoma tissues when compared to matched non-cancer tissues (Figure 1). Approximately 84.3% (59/70) of the colorectal carcinomas showed reduced expression levels of miR-451 and upregulation/overexpression was noted in only 7.1%, (5/70) of patients. The remaining patients (n=4) did not show any change in expression levels for miR-451. The mean expression fold change for miR-451 after normalising with matched non-tumour tissues was 0.46 (ranging from 0.01 to 2.78) with SD of 0.61 (i.e. 0.46 ± 0.61).

Down regulation of miR-451 was noted to be predominant in colorectal carcinoma with co-existing adenoma(s) when compared to cancers without co-existing colorectal adenoma(s) (91.4%, 32/35 versus 77.1%, 27/35, $p = 0.02$). All the cases with high expression of miR-451 did not have co-existing adenoma(s) (0%, 0/5).

On clinical follow-up, cancer recurrence or persistence of cancer after surgery was detected in 29% (20/70) of the patients. All these cancers occurred within 40 months after surgery. Distant metastases (most often in either liver or omentum) were present in all these cases.

In patients with cancer recurrence or persistent disease after surgery (n=20), approximately 80% of the patients had reduced levels of miR-451 expression. Recurrence of cancer was not reported in any case with high miR-451 expression ($p=0.028$). There is no significant correlation between miR-451 expression and age, gender, presence of HNPCC of the patients as well as size, grades, pathological stages, presence of lymphovascular permeation, peri-neural invasion and MSI status of the colorectal carcinoma ($p > 0.05$).

Correlation between miR-451 downregulation and MIF protein expression (table2)

MIF protein was noted predominately in the nuclei of the colorectal carcinoma (Figure 2). In the 10 cases with lowest expression of miR-451, 8 cases (80%, 8/10) expressed high level of MIF protein (score = 2 or 3). Amongst these 8

cases, MIF protein showed nuclear staining in 5 cases (62.5%), the other 3 (37.5%) cases showed both nuclear and cytoplasmic localization.

DISCUSSION

In colorectal cancer, downregulation of miR-451 has been reported previously.[9,15,25] In the current study, we used wider sample size to confirm that miR-451 was down-regulated in colorectal cancer tissues. Our findings support the role of miR-451 as a tumour suppressor gene in colorectal cancer and further point its possible role in cancer development.

It is understood that progression from adenoma to carcinoma is important steps in development of colorectal cancer. Yin and colleagues have reported some down regulation of miR-451 in small population patients with colorectal adenoma and more in colorectal adenocarcinoma.[26] Our study found that there is significant down regulation of miR-451 expression in colorectal carcinoma with co-existing adenoma. These findings may imply that miR-451 downregulation is involved in the development of colorectal adenoma which is in the early steps of pathogenesis of colorectal carcinoma.

The current study demonstrated a significant relationship between miR-451 downregulation and recurrence of colorectal cancer after surgery. By studying circulating miRNAs in blood samples of 30 patients diagnosed with early stage colon cancer, Shivapurkar and colleagues identified miR-451 amongst the 6 panel miRNAs (miR-15a, miR-103, miR-148a, miR-320a, miR-451, miR-596) which can be used to predict the recurrence of early stage colon cancer.[27] Also, significant correlation of miR-451 downregulation to recurrence of tumour had been identified in gastric cancer.[15] In addition, the patients with low miR-451 showed worse overall survival rates than patients with high expression of miR-451 in gastric cancer.[28] In contrast, Brenner et al. stated that miR-451 overexpression has strong relationship with cancer recurrence and worse survival in gastric cancer patients.[29] The discrepancy in these results may attribute to the difference in study population and the percentage of the tumour in the selected samples.

Our results showed no significant relationship between the expression pattern of miR-451 and clinicopathological parameters like age, gender of the patients as well as pathological stage, grade, size, lymphovascular permeation, perineural invasion, MSI status of the tumour. These findings were in concur with those

of Liu and colleagues who studied the expression pattern of miR-451 in nasopharyngeal carcinoma and found no relationship between expression of miR-451 with age, gender of the patients as well as pathological stage, grade and size of the cancer.[13]

MIF has been implicated in carcinogenesis of different types of cancers by inactivating the p53 signalling, [30] or promoting angiogenesis.[31,32] In relation to colorectal cancer, MIF was involved in development, maintenance and metastasis.[33-35] MIF was proven to have direct regulatory roles on several genes in colorectal cancer including COX2 (cyclooxygenase 2) [16], VEGF (Vascular endothelial growth factor) and MMP-9(Matrix metalloproteinase 9).[33] Recently, MIF was reported to have important role in promoting epithelial mesenchymal transition (EMT) in colorectal cancer.[34]

Previous studies have identified MIF gene as a direct target for miR-451.[15-16] The current study has analysed the expression pattern of MIF protein in conjunction with the expression patterns of miR-451 to correlate and confirm their target association in human cancer tissues. Our results for the first time identified the target association of MIF with miR-451 using immunohistochemical analysis. These results are in concurrence with the findings of Bandres and colleagues who found that there is a significant inverse relationship between miR-451 expression and MIF gene in patients with gastric cancer.[15] Thus, it can be hypothesised that miR-451 plays a key role in the pathogenesis of CRC by regulating the expression level of MIF.

In this study, immunohistochemical study showed nuclear localization of MIF in most of the cases. However, our sample size is small. Previous data mostly reported cytoplasmic expression of MIF protein.[33,36,37] Furthermore, both cytoplasmic and nuclear expression had been reported in some studies with predominance of cytoplasmic expression. Although the cytoplasmic expression of MIF protein had been reported in all the cases of lung adenocarcinoma, MIF expression also noted in 79.7% of these cases in the nucleus.[38] Intracellular localization of the MIF may play a role in tumour progression. Kamimura et al. found that the patients with lung adenocarcinoma who show only MIF cytoplasmic

localization had shorter survival times than those with positive MIF expression in the nuclei.[38]

In this study, the downstream effect of miR-451 on MIF protein expression is demonstrated in colorectal cancer. Larger samples of colorectal cancer are needed to document the downstream effect of MIF in the pathogenesis of colorectal cancer. It is worth noting that MIF is not the only target for miR-451. c-myc, P13K/AKT pathway, RAB14, CAB39 have been proposed to be the downstream targets of the miRNA having roles in pathogenesis of cancer. Also, MDR1, Cox-2 and 14-3-3 ζ have been involved as downstream target for miR-451 in the drug resistance in cancer cells. [39]

In summary, this study for the first time correlated the expression pattern of miR-451 to several clinical and pathological features of colorectal carcinoma. The results demonstrated that miR-451 could play a role in early steps carcinogenesis. Also, detection of miR-451 could potentially assist in predication of cancer recurrence. Further research with increase numbers of the patients is necessary to confirm these finding.

Figure legends

Figure 1: **The expression pattern of miR-451 in colorectal cancer tissues:** **(A)** The figure shows that miR-451 was downregulated in most of the cases (59/70). Up regulation of miR-451 was reported in 5 cases only included in this study. The last 6 cases showed no fold change between cancers and matched normal tissues. **(B)** The figure shows the extent of miR-451 downregulation. The patients with miR-451 downregulation were divided to two groups according to our results. One group with fold change between 0.7 - 0.1 whereas the other group with fold change between 0.1-0.01. Most of the cases (37) show fold changes less than 0.1.

Figure 2: **Expression of MIF in colorectal adenocarcinoma with low expression of miR-451.**

2A. Strong staining of MIF protein was noted in adenocarcinoma (x2).

2B. Higher magnification showing the staining was predominately in the nuclei of the adenocarcinoma (x 10)

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Take home message

- miR-451 was downregulated in majority of the colorectal cancer.
- Persistence of cancer or cancer recurrence after surgery correlates with downregulation of miR-451 in colorectal cancer.
- Low expression of miR-451 showed high levels of MIF protein expression in colorectal cancer.

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Table 1

The correlation of miR-451 expression level with different clinical and pathological parameters in patients with colorectal carcinoma.

Parameters	Total no.	High expression	Low expression	Normal expression	p-value
.....					
<u>Age</u>					
≤60	14(20.0%)	2 (14.3%)	11(78.6%)	1(7.1%)	0.507
>60	56 (80.0%)	3 (5.4%)	48 (85.7%)	5 (8.9%)	
<u>Gender</u>					
Male	35 (50.0%)	1(2.9%)	32 (91.4%)	2 (5.7%)	0.236
Female	35 (50.0%)	4(11.4%)	27 (77.1%)	4(11.4%)	
<u>Size</u>					
≤ 40mm	39(55.7%)	2(5.1%)	33(84.6%)	4(10.3%)	0.673
> 40 mm	31(44.3%)	3(9.7%)	26(83.9%)	2(6.5%)	
<u>Location</u>					
Colon	54(77.1%)	5(9.3%)	45(83.3%)	4(7.4%)	0.391
Rectum	16(22.9%)	0(0.0%)	14(87.5%)	2(12.5%)	
<u>Grade</u>					
I	9(12.9%)	0(0.0%)	9(100.0%)	0(0.0%)	0.340
II	52(74.3%)	5(9.6%)	41(78.8%)	6(11.5%)	
III	9(12.9%)	0(0.0%)	9(100.0%)	0(0.0%)	
<u>Stage</u>					
I / II	41(58.6%)	4(9.8%)	35(85.4%)	2(4.9%)	0.282
III/IV	29(41.4%)	1(3.4%)	24(82.8%)	4(13.8%)	
<u>Associated adenoma</u>					
Yes	35(50.0%)	0(0.0%)	32(91.4%)	3(8.6%)	0.025
No	35(50.0%)	5(14.3%)	27(77.1%)	3(8.6%)	
<u>Lymphovascular permeation</u>					
Yes	15(21.4%)	0(0.0%)	14(93.3%)	1(6.7%)	0.443
No	55(78.6%)	5(9.1%)	45(81.8%)	5(9.1%)	
<u>Peri-neural infiltration</u>					
Yes	6(8.6%)	1(16.7%)	4(66.7%)	1(16.7%)	0.455
No	64(91.4%)	4(6.3%)	55(85.9%)	5(7.8%)	
<u>MSI status</u>					
Yes	12(17.1%)	2(16.7%)	9(75.0%)	1(8.3%)	0.370
No	58(82.9%)	3(5.2%)	50(86.2%)	5(8.6%)	
<u>HNPCC</u>					
Presence	6(8.6%)	0(0.0%)	6(100.0%)	0(0.0%)	0.542
Not detected	64(91.4%)	5(7.8%)	53(82.8%)	6(9.4%)	
<u>Cancer recurrence</u>					
Yes	20(28.6%)	0(0.0%)	16(80.0%)	4(20.0%)	0.028
No	50(71.4%)	5(10.0%)	43(86.0%)	2(4.0%)	

Table 2

The immunohistochemical expression and localization in 10 colorectal cancer patients with lowest expression of miR-451

Expression level of miR-451(fold change)	MIF score	MIF protein location
.....		
0.013	3	Nuclear+ cytoplasmic
0.013	1	Cytoplasmic
0.020	2	Nuclear
0.022	3	Nuclear
0.027	3	Nuclear
0.032	2	Nuclear+ cytoplasmic
0.042	2	Nuclear
0.046	0	-----
0.045	2	Nuclear +cytoplasmic
0.056	2	Nuclear